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Novel assay for the detection of CRP protein in rabbit leukocytes using flow cytometry

Nová metóda detekcie CRP proteínu na králičích leukocytoch pomocou prietokovej cytometrie

Jaromír VAŠÍČEK[1,2*], Andrej BALÁŽI[1], Ľubomír ONDRUŠKA[1] and Vladimír PARKÁNYI[1]

[1] Research Institute for Animal Production Nitra, National Agricultural and Food Centre, 951 41 Lužianky, Slovak Republic

[2] Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, 949 76 Nitra, Slovak Republic, *correspondence: jaromir.vasicek@gmail.com

Abstract

C-reactive protein (CRP) is an acute phase serum protein secreted by liver hepatocytes. Besides its presence in the serum, CRP was also found on the surface of human leukocytes. However, the binding ability of CRP to rabbit leukocytes has never been previously investigated. The objective of this study was to optimize the detection of rabbit CRP binding leukocytes in order to observe the acute phase of immune response by flow cytometry after Complete Freund´s Adjuvant (CFA) administration. Blood samples were analysed using ELISA assay and flow cytometry immediately before and 2 days after the CFA administration. Significant ($P<0.01$) increase in the proportion of CRP^+ leukocytes (up to 10%) and in their subsets (lymphocytes up to 14% and granulocytes up to 8%) were observed in samples after CFA immunization. ELISA also revealed significantly ($P<0.01$) higher CRP concentration in rabbit blood plasma after CFA immunization (about 3 mg/L) compared to control samples (about 1.5 mg/L) before immunization. In conclusion, the increase in level of CRP protein during the immune response in rabbits could be measured beside the ELISA also using flow cytometry via CRP binding leukocytes. This novel assay could be therefore successfully applied in further biomedical and veterinary research.

Keywords: C-reactive protein, Complete Freund's adjuvant, ELISA, flow cytometry, rabbit leukocytes

Abstrakt

C-reaktívny proteín (CRP) je sérový proteín akútnej fázy vylučovaný pečeňovými hepatocytmi. Popri jeho výskyte v sére bolo zistené, že CRP sa nachádza aj na povrchu ľudských leukocytov. Avšak, schopnosť CRP viazať sa na králičie leukocyty,

nebola doteraz preskúmaná. Cieľom našej štúdie bolo optimalizovať detekciu králičích leukocytov viažucich CRP pomocou prietokovej cytometrie za účelom sledovania akútnej fázy imunitnej odpovede vyvolanej aplikáciou Kompletného Freundovho adjuvansu (CFA). Vzorky krvi boli analyzované pomocou ELISA metódy a prietokovou cytometriou ihneď pred a 2 dni po aplikácii CFA. Vo vzorkách po CFA imunizácii bol zaznamenaný signifikantný (P<0,01) nárast v podiele CRP^+ leukocytov (do 10%) ako aj u ich subpopulácií (u lymfocytov do 14% a granulocytov do 8%). ELISA analýza rovnako odhalila signifikantne (P<0,01) vyššiu koncentráciu CRP v krvnej plazme králikov po CFA imunizácii (okolo 3 mg/L) v porovnaní s kontrolnými vzorkami pred imunizáciou (okolo 1,5 mg/L). Záverom, nárast hladiny CRP proteínu počas imunitnej reakcie u králikov môže byť okrem ELISA metódy merateľný aj pomocou prietokovej cytometrie prostredníctvom leukocytov viažucich CRP. Táto nová metóda by mohla byť úspešne aplikovaná v ďalšom biomedicínskom a veterinárnom výskume.

Kľúčové slová: C-reaktívny proteín, ELISA, Kompletný Freundov adjuvans, králičie leukocyty, prietoková cytometria

Introduction

C-reactive protein (CRP) is an acute phase serum protein secreted by liver hepatocytes and a member of the pentraxin family of proteins (Osmand et al., 1977). CRP is involved in host defence, regulation of inflammation, and modulation of autoimmune disease (Stein et al., 2000). Human serum concentrations of CRP increase from less than one to hundreds of micrograms per millilitre as part of the inflammatory response to infection or acute injury (Bharadwaj et al., 1999) and thus can be easily measured via ELISA assay (Alexandrov et al., 2015). Besides its presence in the serum, CRP was also found on the surface of human lymphocytes (Baum et al., 1983; Ikuta et al., 1986; Bray et al., 1988) and monocytes (Kolb-Bachofen et al., 1995) or rat liver macrophages (Kempka and Kolb-Bachofen, 1985; Kempka et al., 1990) and mouse macrophages (Zahedi et al., 1989). CRP binding to human leukocytes can be detected using specific antibodies by flow cytometry (Bharadwaj et al., 1999).

Rabbit (*Oryctolagus cuniculus*) is one of the animal species often used as an experimental model in human and veterinary research. The rabbit model served for the study of infectious diseases such as syphilis (Gamboa and Miller, 1984), tuberculosis (Dannenberg, 1991), human T lymphotropic virus-I (Sawasdikosol et al., 1993) and human immunodeficiency virus (Filice et al., 1988). Rabbit is also useful for studies of various non-infectious diseases such as atherosclerosis (Jayo et al., 1994) or eye disorders (Peiffer et al., 1994) and is still the animal of choice for production of many polyclonal antibodies (Mage, 1998). Moreover, rabbits are used in toxicity studies that evaluate the vaccine safety in which CRP could be used as a measureable inflammatory biomarker via ELISA analyses of rabbit serum (Destexhe et al., 2013). However, the binding ability of CRP to rabbit leukocytes has never been previously investigate using flow cytometry.

An antigen injected into the mammal triggers an immune response. The induction of effective immune response to antigens in experimental animals usually requires that the antigen is administered with a potent adjuvant. For this purpose Freund's adjuvant (FA) has been used in laboratory animals for decades. Complete Freund's adjuvant (CFA) is composed of a light mineral oil, mannide monooleate (a surfactant agent), and heat-killed and dried mycobacterial cells. Incomplete Freund's adjuvant (IFA) differs from CFA in that it lacks the killed mycobacterial cells (Stills, 2005).

The objective of this study was to optimize the detection of rabbit CRP binding leukocytes in order to observe the acute phase of immune response using flow cytometry after CFA administration.

Materials and methods

Animals

Young (2 months old) and clinically health crossbreed rabbits of a line based on New Zealand white rabbits (n = 10) reared in air-conditioned hall of a local rabbit farm at NAFC-RIAP Nitra were used in the experiments. The rabbits were not immunized with any vaccine before this experiment. The animals were housed in individual cages under constant conditions and fed ad libitum with a commercial diet as described previously (Ondruska et al., 2016). The treatment of the animals was approved by the State Veterinary and Food Administration of the Slovak Republic no. SK CH 17016 and SK U 18016 in accordance with ethical guidelines presented in Slovak Animal Protection Regulation, RD 377/2012, which conforms to European Union Regulation 2010/63.

Blood collection and immunization

Blood samples were collected from *a. auricularis centralis* to tubes with anticoagulant EDTA (flow cytometry) and heparinized tubes (ELISA) immediately before the subcutaneous administration of Complete Freund´s Adjuvant (125 µl of CFA per kilogram of body weight; Sigma-Aldrich, Bratislava, Slovak Republic) and 2 days after the CFA administration.

Flow cytometry

Whole blood samples in EDTA were lysed using hemolytic solution and washed as described by Faldyna et al. (2001). Cells were then divided into prepared tubes and stained with guinea pig polyclonal FITC-conjugated antibody to rabbit CRP (at dilution 1:50; Cloud-Clone Corp., Texas, USA) according to the producer's manual. The common leukocyte antigen CD45 and CD14 expression was used for the "lymphogate" setup in order to distinguish rabbit lymphocytes from the monocytes as described previously (Vasicek et al., 2015). In each sample, 50,000 cells were measured using flow cytometer FACS Calibur (Becton Dickinson, CA, USA). Dead cells and debris were excluded from the analysis.

ELISA assay

The analysis was performed as described in the previous study (Ondruska et al., 2016). Briefly, the heparinized tubes with blood were centrifuged for 20 min at 850 × g for plasma isolation. The levels of rabbit CRP protein in the blood plasma were quantified using a commercial rabbit ELISA kit (SunRed Bio, Shanghai, China).

Statistical analyses

Observed results were evaluated statistically using t-test in SigmaPlot software (Systat Software Inc., Germany) and expressed as the means ± SEM. P-values at $P<0.05$ were considered as statistically significant.

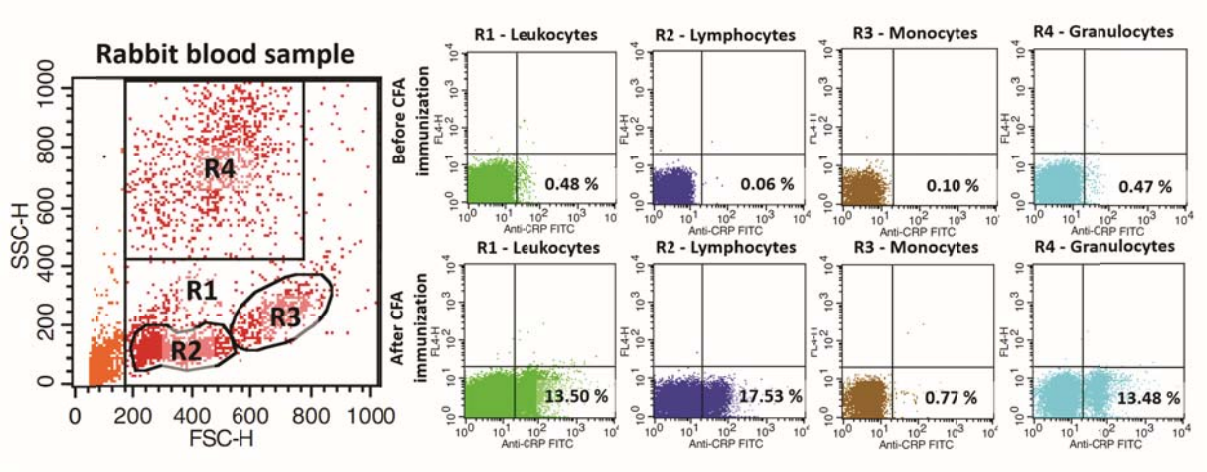
Results

No leukocytes that have bound the CRP protein in the control samples before immunization with CFA were observed (Table 1). On the other hand, significant ($P<0.01$) increase in the proportion of CRP^+ leukocytes (up to 10%) were observed in samples after CFA immunization. Moreover, after immunization significantly ($P<0.01$) increased positivity for CRP binding in almost all leukocyte subsets (lymphocytes up to 14% and granulocytes up to 8%) except for monocytes (about 0.5%; Table 1, Figure 1) was noticed. ELISA analysis also revealed significantly ($P<0.01$) higher concentration of CRP in rabbit blood plasma samples after CFA immunization (about 3 mg/L) in comparison to control samples (about 1.5 mg/L) collected before immunization (Table 1).

Table 1. Proportion of CRP binding leukocytes and concentration of CRP in rabbit blood before and after CFA immunization

Evaluation method		Flow cytometry				ELISA
Blood samples (MEAN ± SEM)	Blood collection	CRP^+ Leuko (%)	CRP^+ Lympho (%)	CRP^+ Mono (%)	CRP^+ Granulo (%)	CRP conc. (mg/L)
	BCI	0.75 ± 0.21[a]	0.07 ± 0.02[a]	0.27 ± 0.15	0.65 ± 0.2[a]	1.59 ± 0.29[a]
	ACI	9.60 ± 2.58[b]	13.85 ± 4.44[b]	0.48 ± 0.08	8.16 ± 2.25[b]	2.94 ± 0.31[b]

BCI – Before CFA immunization, ACI – After CFA immunization, CFA – Complete Freund's adjuvant, CRP^+ – CRP positive cells, Leuko – leukocytes, Lympho – lymphocytes, Mono – monocytes, Granulo – granulocytes, conc. – concentration; [a] vs [b] within the same column were statistically significant at $P<0.01$.



R1 – All leukocytes; R2, R3 and R4 – leukocyte subsets (lymphocytes, monocytes and granulocytes, respectively).

Figure 1. Illustrative dot plots showing evaluating method of CRP positive rabbit leukocytes before and after CFA immunization

Discussion

In the present study, CFA was administrated to juvenile rabbits in order to trigger the native immune response. Any other vaccination was not performed before as the previous study revealed that the level of CRP in rabbit blood plasma decreased significantly after myxoma virus vaccination (Ondruska et al., 2016). Blood samples collected before the CFA administration were used as control samples in order to detect CRP level and proportion of CRP binding leukocytes in rabbits under normal physiological conditions. CRP concentration measured by ELISA in control samples was about 1.5 mg/L. Similar plasma CRP level (about 2 mg/L) were observed in healthy juvenile rabbits in the previous study (Ondruska et al., 2016). This confirmed the normal physiological status of rabbits used in this experiment, since after triggering the immune response by CFA administration ELISA showed approximately twofold increase in the level of CRP (Table 1).

CRP protein was originally identified as a clinical marker of inflammation in humans (Clos and Mold, 2003). Similarly, CRP plasma level assessed via ELISA assay could be used as inflammatory biomarker in toxicity study with rabbits (Destexhe et al., 2013) or as biomarker of health status of rabbit does selected for litter size (García et al., 2012; Ferrian et al., 2013). Furthermore, CRP also activates cells of the immune system through its capacity to bind Fcγ receptors (FcγR); the receptors for IgG, therefore providing a bridge between innate and adaptive immunity. The binding of CRP to FcγR also mediates anti-inflammatory effects (Mold et al., 2002).

In the proposed study, flow cytometry revealed that also in rabbits CRP binds to leukocytes during the immune response (Table 1 and Figure 1). Similar observation were made previously in human leukocyte subsets were CRP was found on lymphocytes (Bray et al., 1988), monocytes and neutrophils (Ballou and Cleveland, 1991; Bharadwaj et al., 1999; Stein et al., 2000). Moreover, Gershov et al. (2000) noticed that CRP also binds to apoptotic cells and so enhance their opsonisation and

phagocytosis by macrophages during inflammatory state. Although, the rabbit monocytes were negative for CRP binding, lymphocytes and granulocytes were highly ($P<0.01$) positive for CRP protein (Table 1 and Figure 1). However, further analyses are required in order to closer specify which type of granulocytes (neutrophils or others) possess the CRP receptor or to assess the proportion of CRP binding apoptotic cells in rabbits.

Nevertheless, these novel research data provide an information about the possibility to measure the proportion of rabbit CRP binding leukocytes using flow cytometry. This could be due to the fact that at the present there is no available flow cytometric antibody specific against rabbit CRP protein at the market. The anti-rabbit CRP antibody used in this study was originally designated for WB, IHC, ICC or IF application, but not for the flow cytometry. Thus, present study demonstrated a successful use of this antibody also for flow cytometry application in rabbits.

Conclusions

The increase in level of CRP protein during the immune response to CFA administration in rabbits could be successfully measured beside the ELISA assay also using flow cytometry via CRP binding leukocytes. This novel assay could be therefore successfully applied in further biomedical and veterinary research.

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